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NOVEL PEPTIDES SUITABLE FOR USE IN ANTIGEN SPECIFIC IMMUNOSUPPRESSIVE THERAPY

FIELD OF THE INVENTION

The invention relates to peptides and their use in treatment of chronic destruction of articular cartilage in autoimmune diseases, pharmaceutical compositions comprising said peptides, a diagnostic method for the detection of autoreactive T cells in a test sample and test kits to be used in said method.

BACKGROUND OF THE INVENTION

The immune system is established on a principle of discrimination between foreign antigens (non-self antigens) and autoantigens (self antigens, derived from the individuals own body) achieved by a build in tolerance against the autoantigens.

The immune system protects individuals against foreign antigens and responds to exposure to a foreign antigen by activating specific cells such as T- and B lymphocytes and producing soluble factors like interleukins, antibodies and complement factors. The antigen to which the immune system responds is degraded by the antigen presenting cells (APCs) and a fragment of the antigen is expressed on the cell surface associated with a major histocompatibility complex (MHC) class II glycoprotein. The MHC-glycoprotein-antigen-fragment complex is presented to a T cell which by virtue of its T cell receptor recognizes the antigen fragment conjointly with the MHC class II protein to which it is bound. The T cell becomes activated, i.e. proliferates and/or produces interleukines, resulting in the expansion of the activated lymphocytes directed to the antigen under attack (Grey et al., Sci. Am., 261:38-46, 1989).

Self antigens are also continuously processed and presented as antigen fragments by the MHC glycoproteins to T cells (Jardetsky et al., Nature 353:326-329, 1991). Self recognition

thus is intrinsic to the immune system. Under normal circumstances the immune system is tolerant to self antigens and activation of the immune response by these self antigens is avoided.

When tolerance to self antigens is lost, the immune system becomes activated against one or more self antigens, resulting in the activation of autoreactive T cells and the production of autoantibodies. This phenomenon is referred to as autoimmunity. As the immune response in general is destructive, i.e. meant to destroy the invasive foreign antigen, autoimmune responses can cause destruction of the body's own tissue.

The contribution of T cells to autoimmune diseases has been established by several studies. In mice, experimental autoimmune encephalomyelitis (EAE) is mediated by a highly restricted group of T cells, linked by their specificity for a single epitope of myelin basic protein (MBP) complexed to an MHC class II molecule. In the Lewis rat, a species with high susceptibility to various autoimmune diseases, disease has been shown to be mediated by T cells.

In humans autoimmune diseases are also thought to be associated with the development of auto-aggressive T cells. A destructive autoimmune response has been implicated in various diseases such as rheumatoid arthritis (RA), in which the integrity of articular cartilage is destroyed by a chronic inflammatory process. The mere presence of cartilage appears necessary for sustaining the local inflammatory response: it has been shown that cartilage degradation is associated with the activity of cartilage-responsive autoreactive T cells in RA (Sigall et al., Clin. Exp. Rheumat. 6:59, 1988; Glant et al., Biochem. Soc. Trans. 18:796, 1990; Burmester et al., Rheumatoid arthritis Smolen, Kalden, Maini (Eds) Springer-Verlag Berlin

Heidelberg, 1992). Furthermore, removal of cartilage from RA patients by surgery was shown to reduce the inflammatory process. The cartilage proteins are therefore considered to be target autoantigens which are competent of stimulating T cells. Activation of these autoreactive T cells leads to development of autoimmune disease. Hence it can be anticipated that functional elimination of these T cells could be beneficial in downregulation of the destructive autoimmune process. However, the identification of the autoantigenic components that play a role in the onset of rheumatoid arthritis has so far remained elusory.

The inflammatory response resulting in the destruction of the cartilage can be treated by various drugs. However, these drugs are immunosuppressive drugs that are nonspecific and have toxic side effects. The disadvantages of nonspecific immunosuppression makes this a highly unfavourable therapy.

Antigen-specific, nontoxic immunosuppression, such as for instance described in WO-A-9510301, provides a very attractive alternative for nonspecific immunosuppression. The antigen-specific therapy involves the treatment of patients with synthetic T cell-reactive peptides which resemble or mimic the epitopes present on the autoantigen. These peptides can therefore be used to induce systemic immunological tolerance, i.e. specific T cell tolerance, both to themselves and to the autoantigen. The induced systemic immunological tolerance is based on the long-observed phenomenon that animals which have been fed or have inhaled an antigen or epitope are less capable of developing a systemic immune response towards said antigen or epitope when said antigen or epitope is introduced via a systemic route. To effectively use the peptide-induced systemic tolerance therapy to treat the T cell mediated cartilage

destruction, there is a great need for T cell-reactive peptides which can desensitize patients against the self antigen that is activating the T cells responsible for the inflammatory process.

SUMMARY OF THE INVENTION

It is an object of the invention to provide peptides which are able to induce systemic immunological tolerance, more in particular specific T cell tolerance, to the responsible cartilage antigen in patients suffering from T cell-mediated cartilage destruction. It is another object of the invention to provide a method for detecting autoreactive T cells involved in the destruction of articular cartilage and test kits to be used in said method.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for such peptides.

In a first aspect of the invention there is provided for peptides consisting of 16 to 55 amino acid residues, said peptide comprising at least one of the amino acid sequences LVCYYTSWS (SEQ ID NO:60), FLCTHIIYS (SEQ ID NO:61), IIYSFANIS (SEQ ID NO:62), LKTLLSVGG (SEQ ID NO:63), FIKSVPPFL (SEQ ID NO:64), FDGLDLAWL (SEQ ID NO: 65), LYPGRRDKQ (SEQ ID NO:66), YDIAKISQH (SEQ ID NO:67), LDFISIMTY (SEQ ID NO:68), FISIMTYDF (SEQ ID NO:69), FRGQEDASP (SEQ ID NO:70), YAVGYMLRL (SEQ ID NO:71), MLRLGAPAS (SEQ ID NO:72), LAYYEICDF (SEQ ID NO:73), LRGATVHRT (SEQ ID NO:74), YLKDRQLAG (SEQ ID NO:75), LAGAMVWAL (SEQ ID NO:76), VWALDLDDF (SEQ ID NO:77) or LDLDDFQGS (SEQ ID NO:78).

In particular, the peptide according to the invention comprises at least one of the amino acid sequences YKLVCYYTSWSQYREG (SEQ ID NO:1), YTSWSQYREGDGSCFP (SEQ ID NO:2), CTHIIYSFANI (SEQ ID NO:5), THIIYSFANISNDHID (SEQ ID NO:6), GWNFGS (SEQ ID NO:12), NTQSRRTFIKSVPPFL (SEQ ID

NO:16), TFIKSVPPFLRTHGFD (SEQ ID NO:17), PPFLRTHGFDGLDLAW (SEQ ID NO:18), HGFDGLDLAWLYPGRR (SEQ ID NO:19), DLAWLYPGRRDKQHFT (SEQ ID NO:20), TIDSSYDIAKISQHLD (SEQ ID NO:28), DIAKISQHLDFISIMT (SEQ ID NO:29), QHLDFISIMTYDFHGA (SEQ ID NO:30), SPLFRGQEDASPDRFS (SEQ ID NO:34), DYAVGYMLRLGAPASK (SEQ ID NO:37), MLRLGAPASKLVMGIP (SEQ ID NO:38), PASKLVMGIPTFGRSF (SEQ ID NO:39), GTLAYYEICDFLRGAT (SEQ ID NO:46), EICDFLRGATVHRTLG (SEQ ID NO:47), RGATVHRTLGQQVPYA (SEQ ID NO:48), VKSKVQYLKDRQLAGA (SEQ ID NO:53), YLKDRQLAGAMVWALD (SEQ ID NO:54), LAGAMVWALDLDDFQG (SEQ ID NO:55), WALDLDDFQGSFCGQD (SEQ ID NO:56) or DFQGSFCGQDLRFPLT (SEQ ID NO:57).

Preferably, the peptide according to the present invention comprises one of the amino acid sequences YKLVCYYTSWSQYREG (SEQ ID NO:1), YTSWSQYREGDGSCFP (SEQ ID NO:2), LDRFLCTHIIYSFANI (SEQ ID NO:5), THIIYSFANISNDHID (SEQ ID NO:6), PNLKTLLSVGGWNFGS (SEQ ID NO:12), QHLDFISIMTYDFHGA (SEQ ID NO:30), SPLFRGQEDASPDRFS (SEQ ID NO:34), DYAVGYMLRLGAPASK (SEQ ID NO:37), MLRLGAPASKLVMGIP (SEQ ID NO:38), YLKDRQLAGAMVWALD (SEQ ID NO:54) or LAGAMVWALDLDDFQG (SEQ ID NO:55).

More preferably, the peptide according to the invention comprises one or more of the amino acid sequences YTSWSQYREGDGSCFP (SEQ ID NO:2), SPLFRGQEDASPDRFS (SEQ ID NO:34), MLRLGAPASKLVMGIP (SEQ ID NO:38), YLKDRQLAGAMVWALD (SEQ ID NO:54) or LAGAMVWALDLDDFQG (SEQ ID NO:55).

The peptides according to the invention consist of 16 to 55, preferably 16 to 35, more preferably 16 to 25, most preferably 16 amino acid residues.

Highly prefered peptides according to the invention are hexadecapeptides consisting of the amino acid sequence YKLVCYYTSWSQYREG (SEQ ID NO:1) YTSWSQYREGDGSCFP (SEQ ID NO:2), LDRFLCTHIIYSFANI (SEQ ID NO:5), THIIYSFANISNDHID (SEQ ID NO:6), PNLKTLLSVGGWNFGS (SEQ ID NO:12), QHLDFISIMTYDFHGA (SEQ ID

NO:30), SPLFRGQEDASPDRFS (SEQ ID NO:34), DYAVGYMLRLGAPASK (SEQ ID NO:37), MLRLGAPASKLVMGIP (SEQ ID NO:38), YLKDRQLAGAMVWALD (SEQ ID NO:54) or LAGAMVWALDLDDFQG (SEQ ID NO:55), more in paricular the amino acid sequences YTSWSQYREGDGSCFP (SEQ ID NO:2), SPLFRGQEDASPDRFS (SEQ ID NO:34), MLRLGAPASKLVMGIP (SEQ ID NO:38), YLKDRQLAGAMVWALD (SEQ ID NO:54) or LAGAMVWALDLDDFQG (SEQ ID NO:55).

Also within the scope of the invention are multimers of the peptides according to the invention such as for example a dimer or trimer of the peptides according to the invention. A multimer according to the invention can either be a homomer, consisting of a multitude of the same peptide, or a heteromer consisting of different peptides.

The characteristic amino acid sequences of the peptides according to the invention can be flanked by random amino acid sequences. Prefered are flanking sequences, that have a stabilizing effect on the peptides, thus increasing their biological availability.

The present invention is based on the unexpected discovery, that Human Cartilage glycoprotein 39 (herein after referred to as HC gp-39) is a target autoantigen in RA patients which activates specific T cells, thus causing or mediating the inflammatory process. HC gp-39 derived peptides were predominantly recognized by autoreactive T cells from RA patients but rarely by T cells from healthy donors, thus indicating that HC gp-39 is an autoantigen in RA. The arthritogenic nature of HC gp-39 was further substantiated in the Balb/c mouse. A single, subcutaneous injection of said protein in Balb/c mice was able to initiate arthritic signs in the animals. The course of the HC gp-39- induced disease was characterized by relapses occuring periodically in fore paws

and/or hind paws and gradually developed from a mild arthritis into a more severe form. Also, a symmetrical distribution of afflicted joints was observed which is, together with the observation of recurrent relapses and nodule formation, reminiscent of disease progression in arthritis, especially RA.

Even more surprisingly it was found that administration of HC gp-39 resulted in immunological tolerance and, more importantly, in delayed and/or suppressed arthritic development.

The amino acid sequences given in SEQ ID NO's 60-78, more specifically the sequences given in SEQ ID NO's 1, 2, 5, 6, 12, 16-20, 28-30, 34, 37-39, 46-48, 53-57 resemble MHC class II restricted T cell epitopes which are present on HC gp-39. Thus, the peptides according to the invention can also be understood to encompass fragments of the autoantigen HC gp-39 which comprise one or more of the above identified MHC Class II restricted T-cell epitopes and they are also within the scope of the invention.

Although HC gp-39 was disclosed in Hakala et al., J.Biol.Chem., Vol.268, No. 34, 25803 (1993), in which it was described as a chitinase protein and suggested for use as a suitable marker for rheumatoid arthritis, any hint or suggestion towards the arthritogenic nature of HC gp-39 was absent.

The peptides according to the invention can be prepared by well known organic chemical methods for peptide synthesis such as, for example, solid-phase peptide synthesis described for instance in J. Amer. Chem. Soc. 85:2149 (1963) and Int. J. Peptide Protein Res. 35:161-214 (1990).

The peptides according to the invention can also be prepared by recombinant DNA techniques. A nucleic acid sequence coding for a peptide according to the invention or a multimer of said peptides is inserted into an expression vector. Suitable

expression vectors are, amongst others, plasmids, cosmids, virusses and YAC's (Yeast Artificial Chromosomes) which comprise the necessary control regions for replication and expression. The expression vector can be brought to expression in a host cell. Suitable host cells are, for instance, bacteria, yeast cells and mammalian cells. Such techniques are well known in the art, see for instance Sambrooke et al, Molecular Cloning:a Laboratory Manual, Cold Spring Harbor laboratory Press, Cold Spring Harbor, 1989.

The peptides according to the invention are T-cell reactive peptides, which are recognized by and are able to stimulate activated, autoreactive T-cells. These autoreactive T cells are found in the blood of RA patients but rarely in healthy donors.

Thus, according to the invention the synthetic peptides, said peptides resembling the MHC Class II restricted T-cell epitopes present on the target autoantigen HC gp-39, are very suitable for use in a therapy to induce specific T-cell tolerance to HC gp-39 in mammals, more specifically humans, suffering from T-cell mediated cartilage destruction, such as for example arthritis, more specifically rheumatoid arthritis.

Although WO 95/01995 and WO 95/02188 describe the diagnostic use of HC gp-39 as a marker for RA, the arthritogenic nature of HC gp-39 is neither disclosed nor suggested. Nowhere do they hint or suggest towards the use of fragments of HC gp-39 or T-cell reactive peptides according to the present invention in the antigen or peptide specific therapy to induce T-cell specific tolerance to the HC gp-39 in the cartilage under attack.

According to the invention, patients suffering from T-cell mediated destruction of the articular cartilage can be treated with a therapeutical composition comprising one or more peptides

according to the invention and a pharmaceutical acceptable carrier. Administration of the pharmaceutical composition according to the invention will induce systemic immunological tolerance, in particular tolerance of the specific autoreactive T cells of these patients, to the autoantigenic proteins in the articular cartilage under attack and other self antigens which display the identified MHC Class II binding T cell epitopes characterized or mimiced by the amino acid sequences of one or more of the peptides according to the invention. The induced tolerance thus will lead to a reduction of the local inflammatory response in the articular cartilage under attack.

Very suitable peptides to be used in a pharmaceutical composition according to the invention are the peptides having 16-55, preferably 16-35, more preferably 16-25, most preferably 16 amino acid residues, said peptides comprising at least one of the amino acid sequences LVCYYTSWS (SEQ ID NO:60), FLCTHIIYS (SEQ ID NO:61), IIYSFANIS (SEQ ID NO:62), LKTLLSVGG (SEQ ID NO:63), FIKSVPPFL (SEQ ID NO:64), FDGLDLAWL (SEQ ID NO: 65), LYPGRRDKQ (SEQ ID NO:66), YDIAKISQH (SEQ ID NO:67), LDFISIMTY (SEQ ID NO:68), FISIMTYDF (SEQ ID NO:69), FRGQEDASP (SEQ ID NO:70), YAVGYMLRL (SEQ ID NO:71), MLRLGAPAS (SEQ ID NO:72), LAYYEICDF (SEQ ID NO:73), LRGATVHRT (SEQ ID NO:74), YLKDRQLAG (SEQ ID NO:75), LAGAMVWAL (SEQ ID NO:76), VWALDLDDF (SEQ ID NO:77) or LDLDDFQGS (SEQ ID NO:78), more in particular one of the amino acid sequences YKLVCYYTSWSQYREG (SEQ ID NO:1), YTSWSQYREGDGSCFP (SEQ ID NO:2), LDRFLCTHIIYSFANI (SEQ ID NO:5), THIIYSFANISNDHID (SEQ ID NO:6), PNLKTLLSVGGWNFGS (SEQ ID NO:12), NTQSRRTFIKSVPPFL (SEQ ID NO:16), TFIKSVPPFLRTHGFD (SEQ ID NO:17), PPFLRTHGFDGLDLAW (SEQ ID NO:18), HGFDGLDLAWLYPGRR (SEQ ID NO:19), DLAWLYPGRRDKQHFT (SEQ ID NO:20), TIDSSYDIAKISQHLD (SEQ ID NO:28), DIAKISQHLDFISIMT (SEQ ID NO:29), QHLDFISIMTYDFIGA (SEQ ID NO:30), SPLFRGQEDASPDRFS (SEQ ID

NO:34), DYAVGYMLRLGAPASK (SEQ ID NO:37), MLRLGAPASKLVMGIP (SEQ ID NO:38), PASKLVMGIPTFGRSF (SEQ ID NO:39), GTLAYYEICDFLRGAT (SEQ ID NO:46), EICDFLRGATVHRTLG (SEQ ID NO:47), RGATVHRTLGQQVPYA (SEQ ID NO:48), VKSKVQYLKDRQLAGA (SEQ ID NO:53), YLKDRQLAGAMVWALD (SEQ ID NO:54), LAGAMVWALDLDDFQG (SEQ ID NO:55), WALDLDDFQGSFCGQD (SEQ ID NO:56) or DFQGSFCGQDLRFPLT (SEQ ID NO:57).

Specifically preferred in a pharmaceutical composition according to the invention are the peptides having 16-55, preferably 16-35, more preferably 16-25, most preferably 16 amino acid residues, said peptides comprising at least one of the amino acid sequences YKLVCYYTSWSQYREG (SEQ ID NO:1), YTSWSQYREGDGSCFP (SEQ ID NO:2), LDRFLCTHIIYSFANI (SEQ ID NO:5), THIIYSFANISNDHID (SEQ ID NO:6), PNLKTLLSVGGWNFGS (SEQ ID NO:12), QHLDFISIMTYDFHGA (SEQ ID NO:30), SPLFRGQEDASPDRFS (SEQ ID NO:34), DYAVGYMLRLGAPASK (SEQ ID NO:37), MLRLGAPASKLVMGIP (SEQ ID NO:38), YLKDRQLAGAMVWALD (SEQ ID NO:54) or LAGAMVWALDLDDFQG (SEQ ID NO:55).

Highly preferred in a pharmaceutical composition according to the invention are peptides having 16-55, preferably 16-35, more preferably 16-25, most preferably 16 amino acid residues, said peptides comprising at least one of the amino acid sequences YTSWSQYREGDGSCFP (SEQ ID NO:2), SPLFRGQEDASPDRFS (SEQ ID NO:34), MLRLGAPASKLVMGIP (SEQ ID NO:38), YLKDRQLAGAMVWALD (SEQ ID NO:54) or LAGAMVWALDLDDFQG (SEQ ID NO:55).

Most preferred in a pharmaceutical composition according to the invention are hexadecapeptides consisting of the amino acid sequence YKLVCYYTSWSQYREG (SEQ ID NO:1) YTSWSQYREGDGSCFP (SEQ ID NO:2), LDRFLCTHIIYSFANI (SEQ ID NO:5), THIIYSFANISNDHID (SEQ ID NO:6), PNLKTLLSVGGWNFGS (SEQ ID NO:12), QHLDFISIMTYDFHGA (SEQ ID NO:30), SPLFRGQEDASPDRFS (SEQ ID NO:34), DYAVGYMLRLGAPASK (SEQ ID NO:37), MLRLGAPASKLVMGIP (SEQ ID NO:38), YLKDRQLAGAMVWALD

(SEQ ID NO:54) or LAGAMVWALDLDDFQG (SEQ ID NO:55), more in paricular the amino acid sequences YTSWSQYREGDGSCFP (SEQ ID NO:2), SPLFRGQEDASPDRFS (SEQ ID NO:34), MLRLGAPASKLVMGIP (SEQ ID NO:38), YLKDRQLAGAMVWALD (SEQ ID NO:54) or LAGAMVWALDLDDFQG (SEQ ID NO:55).

The peptides according to the invention have the advantage that they have a specific effect on the autoreactive T cells thus leaving the other components of the immune system intact as compared to the nonspecific suppressive effect of immunosuppressive drugs. Treatment with the peptides according to the invention will be safe and no toxic side effects will occur.

Systemic immunological tolerance can be attained by administering high or low doses of peptides according to the invention. The amount of peptide will depend on the route of administration, the time of administration, the age of the patient as well as general health conditions and diet.

In general, a dosage of 0.01 to 1000 μg of peptide per kg body weight, preferably 0.5 to 500 μg, more preferably 0.1 to 100 μg of peptide can be used.

Pharmaceutical acceptable carriers are well known to those skilled in the art and include, for example, sterile salin, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil and water. Other carriers may be, for example MHC class II molecules, if desired embedded in liposomes.

In addition the pharmaceutical composition according to the invention may comprise one or more adjuvants. Suitable adjuvants include, amongst others, aluminium hydroxide, aluminium phosphate, amphigen, tocophenols, monophosphenyl lipid A, muramyl dipeptide and saponins such as Quill A. Preferably, the

adjuvants to be used in the tolerance therapy according to the invention are mucosal adjuvants such as the cholera toxine B-subunit or carbomers, which bind to the mucosal epithelium. The amount of adjuvant depends on the nature of the adjuvant itself.

Furthermore the pharmaceutical composition according to the invention may comprise one or more stabilizers such as, for example, carbohydrates including sorbitol, mannitol, starch, sucrosedextrin and glucose, proteins such as albumin or casein, and buffers like alkaline phosphates.

Suitable administration routes are intramuscular injections, subcutaneous injections, intravenous injections or intraperitoneal injections, oral administration and nasal sprays.

The peptides according to the invention are also very suitable for use in a diagnostic method to detect the presence of activated autoreactive T cells involved in the chronic inflammation and destruction of the articular cartilage.

The diagnostic method according to the invention comprises the following steps:

a) isolation of the peripheral blood mononuclear cells (PBMC) from a blood sample of an individual,

b) culture said PBMC under suitable conditions,

c) incubation of said PBMC culture in the presence of one or more peptides according to the invention, and

d) detection of a response of T cells, for example a proliferative response, indicating the presence of activated autoreactive T cells in the individual.

The detection of a proliferative response of T cells can be detected by, for example, the incorporation of ^{3}H-thymidine.

Also within the scope of the invention are test kits which comprise one or more peptides according to the invention. These

test kits are suitable for use in a diagnostic method according to the invention.

The following examples are illustrative for the invention and should in no way be interpreted as limiting the scope of the invention.

EXAMPLES

METHODS

Patients

This study included 7 DR4(DRB1*0401)-positive patients diagnosed as suffering from RA according to the ARA criteria (Arnett et al., (1988), Arthritis Rheum. 31, 315). Peripheral blood samples were obtained with informed consent. There were five women and two men aged 46-79 years. Their duration of disease ranged from over 10 to over 30 years. Three out of 7 patients had at least 3 swollen joints. Four patients did not show any signs of active disease. All patients were on medication: Four patients were treated with prednisone, three patients received anti-rheumatic agents and 4 patients were treated with NSAID's as well.

Peripheral blood samples from 5 healthy donors carrying the DR4(DRB1*0401) specificity were obtained with informed consent and included in this study as a control.

Definition of HLA-DR polymorphisms

Patient and healthy donor peripheral blood mononuclear cells (PBMC) isolated from heparinized peripheral blood by standard centrifugation on Ficoll-Paque were stimulated with PHA (Welcome, Dartford, UK) to obtain 5 x 10^6-10^7 lymphocytes. The

QIA amp blood kit (QIAGEN Inc,) was used to purify chromosomal DNA from cultured cells according to the manufacturers instructions. Chromosomal DNA extracts were analysed using a DR 'low resolution' SSP kit. DR4 subtyping was performed using the Dynal DRB1*04-SSP kit. MHC DR typing was performed at the Transplant Serology Laboratory, University Hospital, Nijmegen, The Netherlands.

Table I

RA Patient	stage	synovitis	duration	HLA-DR
191	IV	no	>30 years	0401/01
259	III-IV	yes	>30 years	0401/16
262	III-IV	yes	>10 years	0401/0408
272	III-IV	no	> 30 years	0401/0701
276	IV	no	>30 years	0401/14
286	IV	no	20 years	0401/0408
287	III-IV	yes	20 years	0401/13
HD				HLA-DR
155				0401/14
157				0401/13
168				0401/07
230				0401/07
235				0401/13

Peptide synthesis

Peptides were synthesized at Eurosequence (Groningen, The Netherlands). Peptides were synthesized from the C-terminus to the N-terminus on a 10 µmol scale using solid-phase FMOC

chemistry. The crude peptides were partly purified by several other preparations. As indicated by the manufacturer, at least 35% of the lyophilized product contained the desired full length product. The rest contained salt and remaining solvent. The quality of the final product was checked by sequence analysis, amino acid analysis and/or RF-HPLC. The sequences of the peptides synthesized are enlisted in Table II.

Table II: Amino acid sequences of the peptides used in this study.

SEQ ID NO:	residu	peptide
1	22-37	YKLVCYYTSWSQYREG
2	28-43	YTSWSQYREGDGSCFP
3	34-49	YREGDGSCFPDALDRF
4	40-55	SCFPDALDRFLCTHII
5	46-61	LDRFLCTHIIYSFANI
6	52-67	THIIYSFANISNDHID
7	58-73	FANISNDHIDTWEWND
8	64-79	DHIDTWEWNDVTLYGM
9	70-85	EWNDVTLYGMLNTLKN
10	76-91	LYGMLNTLKNRNPNLK
11	82-97	TLKNRNPNLKTLLSVG
12	88-103	PNLKTLLSVGGWNFGS
13	94-109	LSVGGWNFGSQRFSKI
14	100-115	NFGSQRFSKIASNTQS
15	106-121	FSKIASNTQSRRTFIK
16	112-127	NTQSRRTFIKSVPPFL
17	118-133	TFIKSVPPFLRTHGFD
18	124-139	PPFLRTHGFDGLDLAW
19	130-145	HGFDGLDLAWLYPGRR

20	136-151	DLAWLYPGRRDKQHFT
21	142-157	PGRRDKQHFTTLIKEM
22	148-163	QHFTTLIKEMKAEFIK
23	154-169	IKEMKAEFIKEAQPGK
24	160-175	EFIKEAQPGKKQLLLS
25	166-181	QPGKKQLLLSAALSAG
26	172-187	LLLSAALSAGKVTIDS
27	178-193	LSAGKVTIDSSYDIAK
28	184-199	TIDSSYDIAKISQHLD
29	190-205	DIAKISQHLDFISIMT
30	196-211	QHLDFISIMTYDFHGA
31	202-217	SIMTYDFHGAWRGTTG
32	208-223	FHGAWRGTTGHHSPLF
33	214-229	GTTGHHSPLFRGQEDA
34	220-235	SPLFRGQEDASPDRFS
35	226-241	QEDASPDRFSNTDYAV
36	232-247	DRFSNTDYAVGYMLRL
37	238-253	DYAVGYMLRLGAPASK
38	244-259	MLRLGAPASKLVMGIP
39	250-265	PASKLVMGIPTFGRSF
40	256-271	MGIPTFGRSFTLASSE
41	262-277	GRSFTLASSETGVGAP
42	268-283	ASSETGVGAPISGPGI
43	274-289	VGAPISGPGIPGRFTK
44	280-295	GPGIPGRFTKEAGTLA
45	286-301	RFTKEAGTLAYYEICD
46	292-307	GTLAYYEICDFLRGAT
47	298-313	EICDFLRGATVHRTLG
48	304-319	RGATVHRTLGQQVPYA
49	310-325	RTLGQQVPYATKGNQW
50	316-331	VPYATKGNQWVGYDDQ

51	322-337	GNQWVGYDDQESVKSK
52	328-343	YDDQESVKSKVQYLKD
53	334-349	VKSKVQYLKDRQLAGA
54	340-355	YLKDRQLAGAMVWALD
55	346-361	LAGAMVWALDLDDFQG
56	352-377	WALDLDDFQGSFCGQD
57	358-373	DFQGSFCGQDLRFPLT
58	364-379	CGQDLRFPLTNAIKDA
59	368-383	LRFPLTNAIKDALAAT

Peptide HLA-DR binding assay

DR4 (DRB1*0401) and DR4 (DRB1*0404) molecules were purified from the homozygous EBV-transformed human B lymphoblastoid cell lines Huly138IC2 and BM92 using the mAb L243, directed against a monomorphic determinant on the DR-complex (Lampson, L.A. and R. Levy (1980), J. Immunol. *125*:293-299).

The peptide binding studies were performed using a semi-quantitative competition binding assay (Joosten et al 1994, Int. Immunol. 6, 751). Briefly, purified HLA-DR molecules (30 nM DR4(DRB1*0401) or 15 nM DR4 (DRB1*0404) were incubated at pH5.0 with 50 nM biotinylated marker peptide (HA $309_{Y\rightarrow F}$) and a concentration range of competitor peptide in a final volume of 25 μl binding buffer (PBS containing 0.01% NaN_3, 0.05% NP-40, 5% DMSO, 1 mM AEBSF, 1 mM N-ethyl maleimide, 8 mM EDTA and 10 μM pepstatin A). After 44 hr of incubation at RT, HLA-DR-bound marker peptide was separated from free marker peptide using a 96 well vacuum dotblot apparatus (Hybri.dot, BRL) and a nitrocellulose membrane (Hybond ECL, Amersham, UK). The nitrocellulose filters were blocked with 0.5% DNA blocking reagent (Boehringer) in 0.1 M maleic acid, 150 mM NaCl, pH7.5.

After 0.5-1 hr, the filters were washed in PBS, 0.05% Tween 20 (Sigma) and incubated with Streptavidin-HRPO (Southern Biotechnology) in a 1:10.000 dilution. Biotinylated peptides were detected by enhanced chemiluminescence using a Western Blot ECL kit (Amersham). Exposure of the preflashed films (Hyperfilm-ECL, Amersham) was for 10 min. The spots were analysed by scanning the films and using Image Quant/Excel software for analysis.

The affinity of a given peptide for binding DRB1*0401-encoded molecules was related to competition with the marker peptide. This relative binding affinity was defined as the peptide concentration at which the signal was reduced to 50% (IC_{50}).

Proliferative responses of blood mononuclear cells

In order to identify T-cell epitopes within HC gp-39, 59 peptides of 16 AA in length, overlapping by 10 AA were tested for their capacity to induce a proliferative response in PBMC from RA patients and healthy controls carrying the DR4 (DRB1*0401) specificity (Table 1). Table 2 enlists the sequences of the peptides tested.

PBMC obtained from heparinized venous peripheral blood were isolated by standard centrifugation on a Ficoll-Paque gradient. Cells were cultured in four-fold at a concentration of $1,5 \times 10^5$ cells / well in medium supplemented with 10% heat-inactivated, autologous plasma, L-glutamine, 2-ME and antibiotics in flatbottomed microtiter plates. Cells were incubated in medium alone or in the presence of phytohaemagglutinin (PHA) (2.5 µg/ml) to assert cell viability, or in the presence of 10 or 100 µg/ml of the HC gp-39-derived peptides. In several cases, sets of 2 or 3 sequential peptides were tested due to limited PBMC numbers of individual donors. Cultures were incubated in a total

volume of 210 µl for 7 days at 37 °C in a humidified atmosphere of 5% CO_2. Cultures were pulsed during the last 18 hours with 0.25 µCi ^{3}H-thymidine ([^{3}H]TdR). Cells were harvested on glassfiber filters and [^{3}H]TdR incorporation was measured by gas scintillation (Packard Matrix 96 βcounter). Only peptides inducing a proliferative response at both 10 and 100 µg/ml were considered to contain a T-cell epitope. Responses were defined positive if stimulation index values (SI, antigen-specific counts per 5min (cp5m)/ background cp5m) exceeded or equaled 2.

RESULTS

Identification of T-cell epitopes by proliferative responses of blood mononuclear cells

T-cell reactivity to HC gp-39-derived peptides was analyzed by measuring the PBMC proliferative response in DR4 (DRB1*0401)-positive RA patients and healthy donors. Proliferative responses were tested in autologous plasma. In Table IIIA and IIIB the results of 7 experiments are presented showing the responses of RA patients (Table IIIA) and the responses of healthy donors (Table IIIB) to 59 overlapping sequences derived from HC gp-39. Donors found to respond to both concentrations (100 and 10 µg/ml) of a peptide were ranked as responders and donors which did not respond to both concentrations tested were ranked as non-responders (NR).

Responses to the individual peptides 1, 2, 5, 6, 12, 15, 30, 34, 37, 38, 40, 41, 54 and 55 (the numbers respond to the respective SEQ ID NO of each peptide, for example, peptide 30 means: peptide having amino acid sequence of SEQ ID NO:30) were

observed in one or more donors, thereby identifying these sequences as T-cell epitopes.

Interestingly, responses to peptides 2, 34, 38, 40, 54 and 55 were observed in RA patients only.

On the other hand, peptides 12 and 41 induced only responses in healthy donors (230, 235) thus far.

In addition, as can be seen in Table 3, responses were found to the following sets of: peptides 1/2, 1/2/3, 4/5/6, 5/6, 15/16, 17/18, 19/20, 28/29/30, 29/30, 37/38, 37/38/39, 39/40, 46/47/48, 53/54, 55/56 and 55/56/57. These results are in accordance with most of the results of the individual peptides mentioned above. Moreover, the responses against the sets of peptides define regions that contain additional T-cell epitopes, i.e. the regions covered by petides 16-20 (residu 112-151), 28-29 (residu 184-205), 38-40 (residu 244-271), 46-48 (residu 292-319) and 53-57 (334-373).

Six out of 7 DR4 (DRB1*0401)-positive RA patients responded to HC gp-39-derived peptides or sets of peptides and were therefore ranked as responders. In the healthy donor group (HD), 3 out of 5 donors were ranked as responders. In general, RA patients appeared to respond to many more HC gp-39 regions than healthy donors (healthy donor 230 being an exception). For example, PBMC from RA patient 272, which were tested against individual peptides, appeared to respond to a total of 11 peptides (1, 2, 5, 6, 30, 34, 37, 38, 40, 54 and 55). PBMC of the other patients (patient 287 being an exception) showed responses against sets of peptides overlapping these 11 sequences and identified some additonal regions containing T-cell epitopes (peptides 14-20 and 46-48).

PBMC derived from a healthy donor (230) further confirmed the presence of T-cell epitopes in peptides 1, 5, 6, 15, 30 and 37.

Overall, the peptides or sets of peptides most frequently recognized contain peptides 1/2, 5/6, 30, 37/38, 54/55).

Correlation of T-cell epitopes and DRB4 (DRB1*0401) binding

Peptides 1, 2, 5, 6, 12, 15, 30, 34, 37, 38, 40, 41, 54 and 55 were all found to stimulate peripheral blood derived T-cells. As a corrollary to this finding, all of these peptides were found to bind to DR4 (DRB1*0401) with relatively high affinity (except peptides 2 and 38 which bind with intermediate relative affinity). Peptides 3, 4, 16, 17, 18, 19, 20, 28, 29, 39, 46, 47, 48, 53, 56 and 57 were tested in sets rather than individualy. It is very likely that several of these peptides also contain relevant T-cell epitopes. In any case, these peptides all can bind DRB4 (DRB1*0401) with high to intermediate relative affinity (except for peptide 20 which binds with poor relative affinity).

Table IIIA: Peptide-induced proliferative responses of PBMC from RA patients.

RA:	272	262	276	286	191	287	259	0401
peptide	0401	0401	0401	0401	0401	0401	0401	binding
	R	R	R	R	R	NR	R	
1	pos							+++
2	pos		pos	pos	pos		pos	+
3								+
4								+
5	pos						pos	+++
6	pos		pos	pos				+++
7								

8								
9								
10								
11								
12								
13								
14&								
15&								+++
16				pos				+++
17								++
18			pos					+
19								+
20				pos				+/-
21								
22								
23								
24								
25								
26								
27								
28								+++
29							pos	+++
30	pos	pos	pos	pos				+++
31								
32								
33								
34	pos							+++
35								
36								
37	pos							+++
38	pos	pos	pos	pos			pos	+
39								++
40&	pos			pos				+++
41&								

42								
43								
44								
45								
46								+++
47					pos			+++
48								+
49								
50								
51&								
52&								
53								+++
54	pos		pos	pos				+++
55	pos							+++
56				pos	pos			+++
57								++
58								
59								
BG	0.2	0.7	0.5	0.8	2.4	0.9	0.2	

*pos = positive responses to both 100 and 10 microgram/ml of peptide or sets of peptides (SI ≥ 2 were regarded positive). Together the peptides (16 AA in length and overlapping by 10 AA) cover the complete mature sequence of mature HC gp-39 (residu 22-383). Peptides were synthesized at Eurosequence (Groningen, The Netherlands). RA = rheumatoid arthritis patient. 0401 = donor carrying the RA-associated HLA-DRB1*0401 specificity. NR = non-responder. R = responder. BG = mean of background counts per 5 minutes x 10 measured in wells without antigen. +++ = high affinity binder (IC50<1 μM); ++ = good affinity binder (1<IC50<10 μM); + = intermediate binder (10<IC50<100 μM); +/- = poor binder (100<IC50<1000 μM); - = non-binder (IC50>1000 μM)*

Table IIIB: Peptide-induced proliferative responses of PBMC from healthy donors.

HD	155	157	168	230	235	0401
peptide	0401	0401	0401	0401	0401	binding
	R	NR	NR	R	R	
1				pos		+++
2						
3						
4						
5				pos		+++
6				pos		+++
7						
8						
9						
10						
11						
12				pos		+++
13						
14&						
15&				pos		+++
16						
17						
18						
19						
20						
21						
22						
23						
24						

25						
26						
27						
28						
29						
30				pos		+++
31						
32						
33						
34						
35						
36						
37	pos			pos	pos	+++
38						
39						
40						
41					pos	+++
42						
43						
44						
45						
46						
47						
48						
49						
50						
51						
52						
53						
54						
55						

56						
57						
58						
59						
BG	4,2	10,4	2,2	3,6	3,5	

pos = positive responses to both 100 and 10 microgram/ml of peptide or sets of peptides (SI ≥ 2 were regarded positive). Together the peptides (16 AA in length and overlapping by 10 AA) cover the complete mature sequence of mature HC gp-39 (residu 22-383). Peptides were synthesized at Eurosequence (Groningen, The Netherlands). HD = healthy donor. 0401 = donor carrying the RA-associated HLA-DRB1*0401 specificity. NR = non-responder. R = responder. BG = mean of background counts per 5 minutes x 10^{-3} measured in wells without antigen. +++ = high affinity binder (IC50<1 μM); ++ = good affinity binder (1<IC50<10 μM); + = intermediate binder (10<IC50<100 μM); +/- = poor binder (100<IC50<1000 μM); - = non-binder (IC50>1000 μM)

ABBREVIATIONS

AEBSF:	4-(2-AminoEthyl)-BenzeneSulfonyl Fluoride
BB:	binding buffer
BCA:	Bicinchoninic Acid
BSA:	bovine serum albumin
DMSO:	Dimethyl Sulfoxide
ECL:	Enhanced Chemiluminescence
EDTA:	EthyleneDiamine Tetra Acetic acid
FACS:	Fluorescence Activated Cell Sorter
HLA:	Human Leukocyte Antigens
HPLC:	High Pressure Liquid Chormatography
HRP:	Horse Radish Peroxidase
MHC CLASS II:	Major Histocompatibility Complex class II

NMR:	Nuclear Magnetic Resonance
NP-40:	Nonidet P-40
PBS:	Phosphate Buffered Saline
PVDF:	Polyvinylidene difluoride
RA:	Rheumatoid Arthritis
SDS-PAGE:	Sodium DodecylSulfate Polyacrylamide Gel Electrophoresis